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Molecular Characterization of HPV16 E6 and E7 Variants among Women with Cervical Cancer in Morocco

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Authors' contributions

Author ZQ carried out the molecular studies, participated in project design and drafted the manuscript. Author MME participated in the conception and design of the study. Author MA participated in sample collection, clinical data acquisition and anatomy pathology analyses. Authors EMEF and MM participated in the sequencing analysis and data analysis. Author MMEK participated in the specimen sampling and in the molecular analysis. Author MEM participated in the design and coordination of the project and reviewed the final manuscript. Author MK participated in the conception and the design of the study. Author MA conceived the study and participated in the design and coordination of the project and drafted the manuscript. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: Human papillomavirus type 16 (HPV16) is the primary etiological agent of cervical cancer. The variations in the amino acid sequence of the HPV16 E6 and E7 oncoproteins are known to correlate with both their oncogenic potential and geographic distribution.

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Study Design: The present study was designed to analyze sequence variations in E6 and E7 genes of HPV16 in order to evaluate the intratype variants circulating in our population.
Methodology: The entire E6 and E7 genes of 31 HPV16 isolates from Moroccan patients with cervical cancer were sequenced and analyzed.
Results: Sequence analysis of HPV16-E6 showed a high prevalence (64.5%) of the African lineage. The European and the North-American variants were detected in respectively 19.4% and 16% of the HPV16 positive specimens. At the amino acid level, the most prevalent missense mutations revealed in the E6 gene were H78Y, Q14D, L83V, R10I and Q14H. Our data also showed that E7 appeared to be better conserved as compared to E6, with a high frequency of two silent variations at G789A and T795C nucleotides and one hot spot of E7 nucleotide variation A647G leading to N29S.
Conclusion: The present study provides a new data on the genetic diversity of HPV16 and highlights the possible association between the high prevalence of HPV16 African variants and the high incidence of cervical cancer in Morocco.

Keywords: Cervical cancer; Human Papillomavirus type 16; E6/E7 variants; Morocco.

1. INTRODUCTION

Cervical cancer is the second most predominant cancer worldwide, and more than 85% of the global burden occurs in developing countries, where it accounts for 13% of all female cancer [1]. In Morocco, the incidence of cervical cancer is the highest in the North African region, with an age-standardized incidence rate (ASR) of 13.5 per 100 000 women [2]. Persistent infection with high risk Human papillomavirus (HPV) is the main etiological factor in the development of cervical cancer. Globally, HPV16, the most frequent HPV type found in cervical cancer, is identified in approximately 65% of cancer cases [3]. In Morocco, our previous reports have shown that the HPV16 is also the most frequent type found in cervical carcinoma [4,5].

Given that the prevalence of cervical cancer varies in different regions and countries, a number of studies have addressed the possible association of HPV16 variant status with different risks for progression to malignancy. The role of HPV intratype gene variations in development of malignancy is an important subject of continued research. Recent studies suggest that variants of HPV 16 can confer an increased risk for developing cervical cancer [6,7]. The early genes of the HPV 16 genome including E2, E4, E5, E6, and E7 are critical in the pathogenesis of HPV-associated cancer, since they regulate several properties such as replication and transcription of viral DNA as well as immortalization and transformation of infected cells. However, E6 and E7 have been extensively studied on their roles in cervical carcinogenesis [8]. The E6 and E7 ORFs, major transforming proteins of HPV16, contain the transforming ability of HPV. E6 oncoprotein destructs p53 and can overcome the p53 protective control pathways [9], which are important in preventing the genetic damage that may lead to cancer. E7 oncoprotein overcome the pRB tumour suppressor Block and binds and activates cyclin complexes [10].

Any change in the sequences of these genes may lead to altered biological function of the proteins encoded by these genes, which in turn may influence the natural history of the infection. Therefore, identification of HPV 16 variants may be important for the rational design of newer diagnostic and therapeutic interventions in cervical cancer as well as for

vaccine development strategies. Thus, further immunological and biochemical analyses focusing on these variable sites are needed [11].

HPV16's genome variants in cervical samples from different geographical areas have been classified into phylogenetic lineages as European (E), Asian (As), Asian-American (AA), African-1 and -2 (Af-1 and Af-2), and North American1 (NA1) [12-14]. Currently, multiple studies have shown that HPV16 variants differ in their association with cervical cancer [7,15,16], viral persistence [6,17,18], the frequency of recurrence of cervical disease [7] and clinical outcome. This phenomenon may be explained by natural variants that alter the immunogenic and/or carcinogenic properties of the virus [19]. Therefore, it appears that the development of malignancy is a consequence of an aberrant host–virus interaction [10].

However, although numerous studies on HPV16 variants in cervical cancer have been carried out so far worldwide, nothing is known concerning HPV variants in patients from Morocco, where there is a significantly high occurrence of this tumor.

Thus, the present study was planned to characterize variants of HPV16 E6 and E7 genes in a sample of Moroccan women with cervical cancer to identify the HPV16 variants circulating in Morocco.

2. MATERIAL AND METHODS

2.1. Clinical Specimens

DNA from thirty one cervical cancer samples determined to be positive for HPV16 in our previous study [4,5] were available from our laboratory DNA bank. Briefly, the majority of cases (96.83%) were diagnosed at advanced stages (IIB and IIIB), whereas one patient (3.13%) was admitted at an earlier stage (IB). All cases were squamous cell carcinoma (SCC) and only one case was a well differentiated adenocarcinoma.

The study was approved by the ethic committee of Pasteur Institute in Morocco and written informed consent was obtained from each study subject.

2.2. Cell Lines

CaSki and SiHa cervical cancer cell lines were obtained from the American Type Culture Collection (ATCC) and used as positive controls. These cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and DNA was isolated using standard methods [20].

2.3. PCR Amplification and DNA Sequencing

HPV16 -E6 and -E7 specific PCRs were performed with primers flanking the encoding region of HPV16E6 ORF (nucleotides 52–575) : 5'-CGAAACCGGTTAGTATAA-3' and 5'-GTATCTCCATGCATGATT-3' and HPV16E7 ORF (nucleotides 480–985) : 5'-ATAATATAAGGGGTCGGTGG-3' and 5'-CATTTTCGTTCTCGTCATCTG-3' [21]. PCR amplifications were performed in a 25 µl volume containing 1x PCR buffer, 1,5mM $MgCl_2$, 100 µM each dNTP, 0,2 µM forward and reverse primers, 100 ng genomic DNA and 0,25 U gold Taq DNA polymerase (Applied Biosystems, USA). The amplification mixtures were first denatured at 94°C for 7 min. Then, thirty-five cycles of PCR were performed with

denaturation at 94°C for 1 min, primer annealing for 1 min at 55°C and primer extension for 1 min at 72°C. At the end of the last cycle, the mixtures were incubated at 72°C for 7 min. For every reaction, positive controls using DNA extracted from SiHa and Caski cell lines, as well as a negative control without template DNA, were included. PCR products were tested on an ethidium bromide stained 2% agarose gel. Positive PCR products were purified by the ExoSaP IT^R clean up system (USB, USA). Sequencing reaction was performed according to the manufacturer's protocol (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, USA). Direct sequencing of amplified PCR products was performed with both forward and reverse primers on an ABI PRISM sequencing apparatus (ABI Prism 3130XL Genetic Analyser, Applied Biosystems, USA) in molecular and functional genomics platform (UATRS-CNRST, Rabat, Morocco). In approximately half of the samples, the PCR amplification and sequencing were repeated to exclude PCR artifacts.

2.4. PCR-Quality Control

To avoid contamination leading to false positive results, all PCR-related work was carried out in specialized zones within a PCR laboratory that undergoes UV purification at least once every 24 hours. To detect crossover contamination, positive controls with DNA extracted from SiHa and Caski cell lines, as well as a negative control (PCR reagents mixture without template DNA) were included in every set of 10 clinical specimens for each PCR run. All negative controls were negative for β-globin and HPV assay. Positive controls containing the SiHa and Caski cell lines were always positive for β-globin and HPV DNA.

2.5. Sequence Analysis and Phylogenetic Relationship

The HPV16 -E6 and -E7 sequences obtained were aligned with those of European prototypes of HPV sequence (HPV16-R) (GenBank accession numbers: K02718, NC_001526) types, available through the GenBank database (NCBI, National Institute of health, Bethesda, MD, USA), using the online BLAST 2.0 software server (http://www.ncbi.nih.gov/BLAST/) and MEGA5 program. Multiple Sequence Alignment (MSA) was done using CLustalW and phylogenetic relationships were built with the UPGMA method using the computer software MEGA5.

3. RESULTS

Successful amplification of E6 and E7 genes was obtained for all the 31 HPV16 positive studied specimens. Nucleotide sequences of the complete E6 and E7 open reading frame of all the 31 HPV16 positive specimens were compared to the HPV16 reference sequence (Accession Number: K02718, NC_001526). Accordingly, HPV16-positive specimens were classified into E, As, AA, Af1 and Af2, or NA1 branches.

3.1. E6 Sequence Variations and Phylogenetic Relationship

The nucleotide changes and variants identified in this study are shown in Table 1. Sequence analysis of all HPV16 positive specimens showed that there was no evidence of premature stop codons, insertions or deletions. Of the 31 HPV 16 E6 DNA positive samples subjected to nucleotide sequencing, 28 (90.3%) samples revealed nucleotide variations in the E6 sequence while 3 (9.7 %) samples revealed the prototype sequence Ep-350T.

After retrieving these sequences, the computer software MEGA5 was used to reconstruct the phylogenetic relationships among the 31 samples. The tree is shown in Fig. 1. The phylogenetic tree illustrates the relationship between the 31 HPV16 variants based on the alignment of 158 amino acids segments of E6 protein. The phylogenetic tree was separated into two principal phylogenetic branches. One branch was formed by 3 variants Af1-b, Af1-a and Af2-a. The other branch contained 2 variants NA-1b and E-350G including the reference and the prototype sequences. In addition, the E6 protein of HPV16 variants showed significant distances between any two variants and between the variants and the reference clone (Fig. 1).

Considering all variants, 13 different point mutations were detected within the E6 segment, of which A131G, G132C, G132T, C143G, G145T, C285G, T295G, C335T and T350G were missense mutations with substitution of Arginine to Glycine (R10G), Arginine to Tyrosine (R10T), Arginine to Isoleucine (R10I), Glutamine to Aspartic acid (Q14D), Glutamine to Histidine (Q14H), Alanine to Glycine (A61G), Aspartic acid to Glutamic acid (D64E), Histidine to Tyrosine (H78Y) and Leucine to Valine (L83V), respectively (Fig. 2). The remaining four mutations, T109C, T286A, A289G, and A403G, lead to silent mutations at codons 2 (Phenylalanine), 61 (Alanine), 62 (Valine) and 100 (Leucine), respectively. The most prevalent missense variants were H78Y (C335T), Q14H (G145T), Q14D (C143G), L83V (T350G), R10I (G132T), R10G (A131G) and D64E (T295G); they were observed respectively in 80.6% (25/31), 80.6% (25/31), 64.5% (20/31), 51.6% (16/31), 29% (9/31), (7/31) 22.6% and (7/31) 22.6% of total samples (Table 1). The other missense variants were R10T (G132C) and A61G (C285G) and were seen in 12.9% (4/31), and 6.4% (2/31), respectively. The remaining four mutations, T286A, A289G, A403G and T109C leading to silent mutations were found respectively in 80.6% (25/31), 80.6% (25/31), 29% (9/31) and 22.6% (7/31) (Table 1).

The 350 T to G missense mutation (L83V) was detected in HPV16 E6 gene of SiHa and CasKi positive controls with an additional mutation 442 A to C (E113D) in SiHa and 131 A to G in CasKi (R10G). These results are consistent with previous studies showing the same mutations in these two cell lines [22].

Among the 31 analyzed HPV16 samples and based on the E6 sequences obtained in this study, the majority, 20 (64.5%), belonged to African lineage (Af): 11 belonging to the Af-1 class (35.5%) and 9 to Af-2 (29%). All Af isolates show a common pattern of five mutation in E6, namely, C143G, G145T, T286A, A289G, and C335T, which give rise to two amino acid changes, Q14D and H78Y. European lineage was detected in six samples (19.4%), with three belonging to the Ep-350T class (9.7%) and three to Ep-350G (9.7%). The NA-1 variants were detected in five (16%) of the specimens. As or AA variants were not detected in the present study (Table 1).

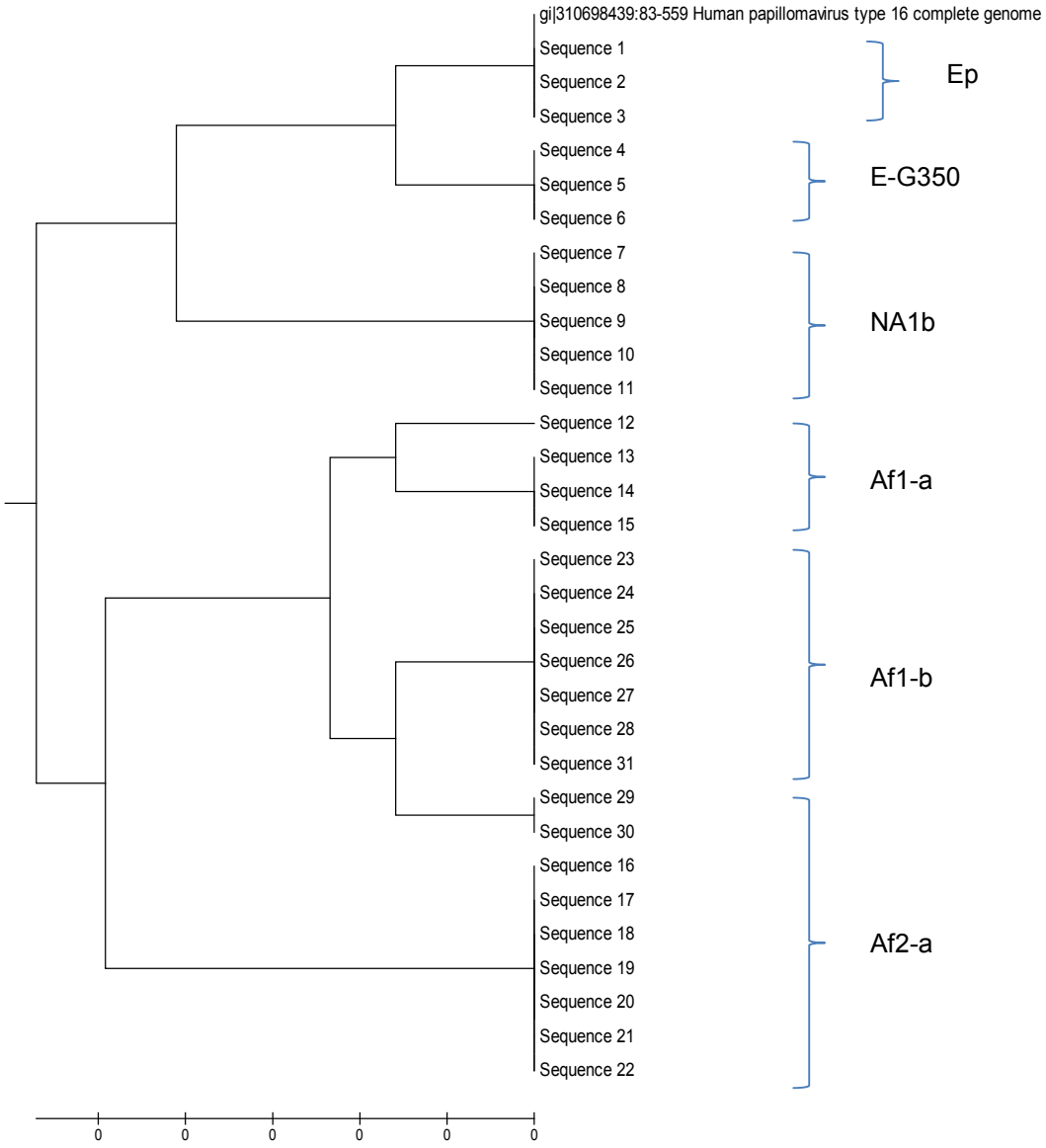

Fig. 1. Evolutionary relationships of 31 HPV16 E6 sequences isolated from Moroccan women with cervical carcinoma.

The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0,05509523 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 32 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 158 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

The Fig. 2 represents amino acid MSA of 31 HPV16 E6/E7 proteins. The variable sites are highlighted. Within the Af branch, the two main subgroups, Af-1 and Af-2, could be distinguished by R10T (Af-1) or R10I (Af-2) substitutions at codon 10. In addition, Af1-b class is characterized by the variant D64E while NA1 branch could be distinguished from the Af branch by the variant Q14H.

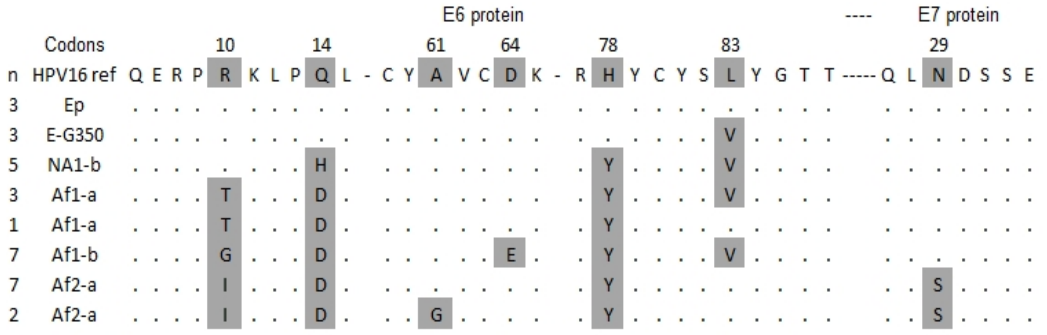

Fig. 2. Amino acid Multiple Sequence Alignment (MSA) showing differences in E6/E7 proteins by region

3.2. E7 Sequence Variations

Compared with the HPV16 reference DNA sequence (Accession Number: K02718, NC_001526), our results showed that the nucleotide variation rate of the full open reading frame of HPV16 E7 region from 31 cervical cancers with HPV16 infection was 67.7% (21 out of 31) for cervical cancer (Table 1). As compared to E6, E7 appeared to be better conserved and revealed only three nucleotide variations. The common silent mutations detected in the E7 gene were T789C (61.3%) and T795G (54.8%) for the amino acids Isoleucine (I) and Threonine (T) at codons 76 and 78, whereas A647G detected in 9 samples (29%) was missense mutation with substitution of Asparagine to Serine (N29S) (Table 1). Based on E7 sequence variations, all nine detected samples harboring the E7 N29S point mutation variants have been subsequently classified as Af-2 variant (Table 1, Fig. 2).

Table 1. Nucleotide sequence variations at E6 and E7 open-reading frames (ORFs) among 31 HPV16 isolates from Morocco

		E6 DNA sequence												E7 DNA sequence			Predicted amino acid substitution	
CLASS	**n**	**109**	**131**	**132**	**143**	**145**	**285**	**286**	**289**	**295**	**335**	**350**	**403**	**647**	**789**	**795**	**E6**	**E7**
HPV16R	-	T	A	G	C	G	C	T	A	T	C	T	A	A	T	T		
Ep-350T	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	prototype	-
Ep-350G	2	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	L83V	-
Ep-350G	1	-	-	-	-	-	-	-	-	-	-	G	-		c	g	L83V	-
NA1b	1	-	-	-	-	T	-	a	g	-	T	G	-	-	-	-	Q14H//H78Y/L83V	-
NA1b	4	-	-	-	-	T	-	a	g	-	T	G	-	-	c	g	Q14H/H78Y/L83V	-
Af1a	1	-	-	C	G	T	-	a	g	-	T	G	-	-	-	-	R10T/Q14D/H78Y/L83V	-
Af1b	1	-	G	-	G	T	-	a	g	G	T	G	-	-	-	-	R10G/Q14D/D64E/H78Y/L83V	-
Af1b	6	-	G	-	G	T	-	a	g	G	T	G	-	-	c	g	R10G/Q14D/D64E/H78Y/L83V	-
Af1a	2	-	-	C	G	T	-	a	g	-	T	-	-	-	-	-	R10T/Q14D/H78Y	-
Af1a	1	-	-	C	G	T	-	a	g	-	T	-	-	-	c	g	R10T/Q14D/H78Y	-
Af2 a	1	c	-	T	G	T	-	a	g	-	T	-	g	G	c	g	R10I/Q14D/H78Y	N29S
Af2 a	2	c	-	T	G	T	-	a	g	-	T	-	g	G	-	-	R10I/Q14D/H78Y	N29S
Af2 a	1	c	-	T	G	T	-	a	g	-	T	-	g	G	c	-	R10I/Q14D/H78Y	N29S
Af2 a	2	c	-	T	G	T	-	a	g	-	T	-	g	G	c	g	R10I/Q14D/H78Y	N29S
Af2a	1	c	-	T	G	T	G	a	g	-	T	-	g	G	c	g	R10I/Q14D/A61G/H78Y	N29S
Af2a	1	-	-	T	G	T	G	a	g	-	T	-	g	G	c	g	R10I/Q14D/ A61G /H78Y	N29S
Af2a	1	-	-	T	G	T	-	a	g	-	T	-	g	G	c	g	R10I/Q14D/H78Y	N29S
Total	31	7	7	13	20	25	2	25	25	7	25	16	9	9	19	17		

The prototype sequence (HPV-16R) is indicated as reference. Phylogenetic lineages are noted as E for European, Af1 for African 1, Af2 for African 2 and NA1 for North-American 1. The number and the letter represent the nucleotide change at that position. Lower letters represents a nucleotide change at that position without an amino acid change. Capital letters indicate variants with an amino acid change. In the 'Predicted substitution' column, the letter preceding the amino acid position refers to the reference HPV16 sequence and the letter after it refer to the substitution. Dashes indicate no variation.

4. DISCUSSION

In the present study, we have examined sequence variations in the HPV16 E6 and E7 genes in Moroccan women with cervical cancer. HPV16 E6 sequencing showed a high diversity of HPV16 genotype in Moroccan patients with cervical cancer. Of the 31 HPV 16 E6 DNA positive samples subjected to analysis, 28 samples (90.3%) revealed nucleotide variations in the E6 sequence while only tree samples (9.7%) revealed the prototype sequence Ep-350T. Our findings are in agreement with published results reporting that approximately 90% of the E6 genes in invasive cervical cancer contained variations [23].

All HPV16 variants detected have been previously described. The majority of HPV16 variants described in this study belong to African branches (64.5%). Our findings are in agreement with published results reporting that the Af lineage are the most frequently detected isolate in Africa [12,24,25] and have been reported to have increased risk for cancer in some [7,26,27] but not all [28] reports. However, as the number of samples analyzed in this study was not high, our data do not address this question.

Within the Af lineage, the two main subgroups, Af-1 and Af-2 were detected in 35.5% and 29% respectively. Our study also confirms the importance of HPV-16 Af-1, the most prevalent of the HPV-16 subtypes in Africa [12,25].

Furthermore, this race-related distribution of HPV16 Af lineage in Moroccan women is consistent with previous findings of geographic-related distributions, whereby the European variants were predominant in populations in Europe, African variants were predominant in Africa, AA variants were predominant in some Asians, American Indians, South and Central America and Spain, while E-As variants were predominant in South-East Asians [25,29].

Our data further show that the European lineage was observed in 19.4% and is in agreement with published results reporting that European lineage was well distributed among the different geographical regions [25]. More surprising was the detection of NA-1 in 16% of this patient population. In this study, the occurrence of NA-1 classes, which is a derivative of the AA and Af-2 variants [12], is difficult to explain. Phylogenetically, HPV variants evolved within local populations of the continents and the possibilities of spread of the molecular variants of HPV 16 from one continent to another have been shown to exist [30]. Recently, the NA-1 lineage was found to be particularly frequent in samples from North Africa during analysis of sequence variations in the HPV 16 positive tumors samples collected globally [25], suggesting that this geographical region represents a previously understudied branch of HPV16 evolution.

The identification of HPV16 Af, E and NA-1 variants also suggests a different sexual mixing behavior and warrants the need for monitoring a wider range of HPV variants involved in cervical cancer in Morocco.

The most frequently observed missense variants of E6 among our patients group were H78Y (C335T), Q14D (C143G) and L83V (T350G), and were detected respectively in 80.6%, 64.5% and 51.6% of analyzed cases. Our finding re-confirm that the dominance of a specific genomic variant is associated with geographic diversity [12,23,31] and some of the mutations identified in the E6 genes of HPV16 isolated in Morocco may have considerable biological impact. The others E6 variants identified among our patient group were, in order of decreasing prevalence, R10I (29%), R10G (22.6%), D64E (22.6%), Q14H (16.1%), R10T (12.9%) and A61G (6.5%). Thereby, the majorities of the E6 amino acid switches in our

samples were located at amino acid residues 10 (R10G, R10I or R10T), 14 (Q14H or Q14D), 78 (H78Y) and 83 (L83V) and were detected respectively in 64.5%, 80.6%, 80.6% and 51.6% of analyzed cases. Interestingly, the three codon sites in the HPV16 E6 open reading frame coding for amino acids 10, 14, and 83 were shown to be under selective pressure [11]. This selection pressure, driven by protein–protein polymorphic interactions, could facilitate the life cycle and pathogenicity of HPV associated disease and might be under immune selection. Certain HLA class I alleles, in concert with specific HPV variants, could be associated with a predisposition for cervical cancer development, whereas others may be protective.[11].It is possible that HPV16 variants in concert with HLA and other immune-genetic polymorphisms play a role in persistence [19]. The immunologic relevance of the HPV16 E6 N-terminal region and variant positions E6 amino acids 10 and 14 is supported by the demonstration of an endogenously processed HLA A*0201-restricted E6 peptide (E6 amino acids 11 to 19) as well as of an overlapping HLA B-7-restricted E6 peptide (E6 amino acids 8 to 15) in this region [32,33]. Indeed, it has been suggested that women with three distinct HLA class I alleles, namely HLA-B*44, HLA-B*51, or HLA-B*57 who were infected with the HPV16 E6 variant L83V had an approximately four to five fold increased risk for cancer [34,35]. Additionally, the HPV 16 E6 variant G131 (R10G) was demonstrated to alter a B*07 binding epitope in such a way that it may influence cytotoxic T lymphocyte's immune recognition [32].

The E6 variants R10G/L83V and Q14H/H78Y/L83V, detected in our study, were more prone to undergo cell-detachment-induced apoptosis than E6 prototype in a model of human normal immortalized keratinocytes (NIKS) [36]. Also, Q14D/H78Y/L83V variants showed statistically significant results with regard to late apoptotic cells. Therefore, the observed phenotype detected in our study may impact on the viral life cycle, which is tightly linked to the differentiation program of the cell.

Our findings are in agreement with the widely reported data demonstrating that the E7 oncoprotein is highly conserved in most populations [37-40] and revealed only three nucleotide variations in 21 (67.7%) patients, whereas the other 10 (22.3%) cases showed the prototype sequence.

Nucleotide sequencing analysis of the E7 gene revealed only one variant (A647G) with substitutions resulting in amino acid change of Asparagine to Serine (N29S) in nine of analysis cases (29%), approximately close to the frequency (36,4%) detected in Tanzania [40]. This mutation was mostly described in Asian countries and display geographical dependence. The reported prevalence of the N29S variant varies: 72.2% for China [41]; 60% for Japan [39]; 59,5% for Korea [42]; 58% for southern china; 14,3% for Sichuan in central China [43] and 0,9% for Germany [38]. Based on E7 sequence variations, all detected samples harboring the E7 N29S point mutation variants have been subsequently classified as Af2 variant. Interestingly, Buonaguro et al. [44] have shown that the E7 N29S variant was absent in all analyzed Ugandan isolates classified as Af-1 variants based on the LCR sequence. A similar study reported that HPV16 isolates harboring the E7 N29S point mutation have been subsequently classified as Af-2 variants on the basis of LCR sequence [40,45]. Therefore, the E7 N29S variant could represent a distinctive feature of the Af-2 lineage.

5. CONCLUSION

The present study has some limitations which must be taken into account: The small number of samples analyzed and the lack of women with HPV 16 from precancerous lesions and

normal cervices in our study population. However, our results show the predominance of HPV16 AF variants that could be associated with cervical cancer, suggesting that the high incidence of cervical cancer in African countries could be associated with the presence of HPV 16 variants. Thus, in-depth studies are needed to support this and to assess the clinical and biological effects of these variants.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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